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REGULAR ARTICLE

The insulin receptor is differentially expressed in somatic and visceral primary sensory neurons

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Abstract

Recent studies demonstrated the expression of the insulin receptor (InsR) and its functional interaction with the transient receptor potential vanilloid type 1 receptor (TRPV1) in primary sensory neurons (PSNs). The present study was undertaken to reveal the target-specific expression of the InsR and its co-localization with the TRPV1 in rat PSNs. We assessed the localization of the InsR and its co-localization with the TRPV1 in PSNs retrogradely labelled with biotin-conjugated wheat germ agglutinin injected into the dorsal hind paw skin, the gastrocnemius muscle, the pancreas and the urinary bladder wall. The largest proportions of retrogradely labelled InsR-immunoreactive neurons were identified among PSNs serving the pancreas (~ 54%) and the urinary bladder (~ 53%). The proportions of retrogradely labelled InsR-immunoreactive neurons innervating the dorsal hind paw skin and the gastrocnemius muscle amounted to ~ 22 and ~ 21%. TRPV1-immunoreactive neurons amounted to ~ 63, ~ 62, ~ 67 and ~ 65% of retrogradely labelled cutaneous, muscle, pancreatic and urinary bladder PSNs, respectively. Co-localization of the TRPV1 with the InsR was observed in ~ 16, ~ 15, ~ 29 and ~ 30% of retrogradely labelled cutaneous, muscle, pancreatic and urinary bladder PSNs. These quantitative immunohistochemical data demonstrate a preponderance of InsR-immunoreactivity among PSNs, which innervate visceral targets. The present findings suggest that visceral spinal PSNs are more likely to be exposed to the modulatory effects of insulin on sensory functions, including neurotrophic, nociceptive and inflammatory processes.

Keywords Insulin receptor · Transient receptor potential vanilloid type 1 receptor · Primary sensory neurons · Retrograde labelling · Somatic and visceral organs

Introduction

Insulin, apart from being a pivotal regulator of body metabolism, is significantly involved in various neuronal processes, such as neuronal survival, initiation of neurite outgrowth and regulation of neuronal activity (Recio-Pinto et al. 1986; Fernyhough et al. 1993; Wan et al. 1997; Barber et al. 2001; Stella et al. 2001). Recently, it has also been revealed that neural actions of insulin are mediated by the InsR, which has been demonstrated in the nerve tissue, too (Sugimoto et al. 2000; Figlewicz 2016). Insulin, at physiological concentrations, enhances neurite outgrowth of cultured primary sensory neurons (PSNs) (Fernyhough et al. 1993; Singh et al. 2012) and exerts trophic effects on a specific subpopulation of PSNs that is sensitive to nerve growth factor (NGF) (Recio-Pinto et al. 1984; Recio-Pinto and Ishii 1988; Fernyhough et al. 1993). In addition, experiments on cultured rat PSNs demonstrated that insulin enhances the capsaicin-induced cobalt uptake resulting from the activation of the nociceptive ion channel, the transient receptor potential vanilloid type 1 receptor (TRPV1) (Sathianathan et al. 2003). TRPV1-expressing PSNs comprise about 60% of C-fibre ganglion neurons and transmit nerve impulses generated by noxious heat and painful chemical stimuli in somatic and visceral organs (Jancsó et al. 1977; Buck and Burks 1986; Holzer 1991; Caterina and Julius 2001). In addition to being essential in the transmission of

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nociceptive information towards the central nervous system, this particular class of PSNs is implicated in the initiation and maintenance of sensory neurogenic vascular and inflammatory reactions collectively termed the neurogenic inflammatory response (Jancsó et al. 1967, 1977; Holzer 1991). Further, it has been reported that nociceptive afferent nerves may also promote secretory, contractile and immune functions in the innervated organs (Maggi and Meli 1988; Holzer 1991; Nagy et al. 2004).

Several studies suggest that interactions among insulin, the InsR and the TRPV1expressed in PSNSs may contribute to physiological and pathophysiological processes including, for example, inflammatory changes of the skin (Gamse et al. 1987; Santicioli et al. 1987), the dura mater (Dux et al. 2007) and the exocrine and endocrine pancreas (Nathan et al. 2001; Razavi et al. 2006; Gram et al. 2007; Tsui et al. 2007). Immunohistochemical studies provided further support to this notion by showing a substantial co-localization of the TRPV1 and the InsR in rat and mouse PSNs of unidentified target innervation territories (Sugimoto et al. 2002; Sántha and Nagy 2006; Baiou et al. 2007) and in rat pancreatic spinal and vagal PSNs (Lázár et al. 2018).

The expression of the InsR in nociceptive sensory nerves of different organs and tissues may bear particular interest as regards the modulatory influence of the ubiquitous metabolic hormone insulin on a variety of tissue processes, in particular on pain and inflammation. Hence, exploring the localization of InsRs in PSNs serving somatic and visceral organs is of critical importance for the further understanding of the role of these particular afferent nerves under physiological and pathological conditions. Therefore, the aim of the present study is to reveal the expression of InsRs in rat cutaneous, muscle, pancreatic and urinary bladder afferent neurons and its co-localization with the TRPV1.

Materials and methods

All experiments were approved by the Ethics Committee for Animal Care at the University of Szeged and were carried out in full accordance with the Directive 2010/63/EU of the European Parliament and of the Council on the Protection of Animals Used for Scientific Purposes and the guidelines of the Committee for Research. All efforts were made to minimise animal suffering. The number of experimental animals was kept as low as possible.

Retrograde labelling of somatic and visceral spinal primary sensory neurons

Adult male Wistar rats ($n = 12$), weighing 300–350 g, were anaesthetised with isoflurane (Forene™, AbbVie Hungary Ltd., Budapest, Hungary). To identify cutaneous, muscle and visceral afferents, biotin-conjugated wheat germ agglutinin (bWGA; Sigma-Aldrich, Gillingham, UK, 1% in distilled water) was injected into the dorsal hind paw skin, the gastrocnemius muscle, the urinary bladder wall and the pancreas using a Hamilton microsyringe. For labelling of dorsal root ganglion (DRG) neurons serving the skin, 2 × 2-μl bWGA solution was injected into the dorsal hind paw skin. The gastrocnemius muscle was exposed through an incision at the dorsal aspect of the calf and 2 × 2-μl bWGA solution was injected into the muscle. To label DRG neurons innervating the pancreas or the urinary bladder, these organs were exposed through a midline abdominal incision and 4 × 2-μl bWGA solution was injected into the parenchyma of the pancreas or the urinary bladder wall. Thereafter, the exposed area was thoroughly rinsed with saline and the wound was closed in layers. After recovery from anaesthesia, the rats were returned to the animal house. Three days later, the rats were anaesthetised with an overdose of thiopental sodium (Insera Arzneimittel GmbH, Freiburg, Germany) (150 mg/kg i.p.) and perfused transcardially first with 100-ml saline, followed by 4% paraformaldehyde in 0.1-M phosphate buffer (pH 7.4). Selected DRGs were removed, post-fixed for 2 h and stored in 0.1-M phosphate buffer (pH 7.4). Twenty-four hours before sectioning, the specimens were transferred into a sucrose-buffer solution.

Serial frozen sections, 15 μm in thickness, were cut on a cryostat and processed for immunohistochemistry. The L_3–L_5 (Gamse et al. 1982; Swett et al. 1991), $L_{4–5}$ (Peyronnard et al. 1986; Swett et al. 1991), L_3-S_1 (Jancsó and Maggi 1987) and $Th_{10–13}$ (Won et al. 1998; Takamido et al. 2006; Lázár et al. 2018) DRGs were used to analyse the proportions of InsR- and TRPV1-immunoreactive neurons, which innervate the dorsal hind paw skin, the gastrocnemius muscle, the pancreas and the urinary bladder, respectively.

Immunohistochemistry

Sections were rinsed in phosphate buffered saline (PBS) for 2 × 10 min, incubated in PBS containing 10% horse serum and 0.1% Triton X-100 for 30 min and processed for staining with the indirect immunofluorescence technique using the following antibodies: rabbit anti-InsR α subunit antibody (Santa Cruz Biotechnologies, Dallas, Tex., USA; 1:500), guinea pig anti-TRPV1 antibody (Neuromics, Edina, Minn., USA; 1:1500). The specificity of these antisera were assessed in DRG sections by the lack of staining with the TRPV1 antibody in specimens obtained from $TRPV1^{-/-}$ mice and by the failure of staining with the InsR antibody after preincubation with the immunising peptide supplied by the manufacturer

(Baiou et al. 2007). Donkey anti-rabbit IgG labelled with DL488 (1:500) and donkey anti-guinea pig IgG labelled with Cy3 (1:500) were used as secondary antibodies (all from Jackson Immunoresearch Laboratories, West Grove, Pa., USA). bWGA lectin was detected by using an extravidine-AMCA conjugate (Jackson Immunoresearch Laboratories). All antibodies were diluted in phosphate buffered saline containing Triton X100 (0.3%). Sections were incubated in the presence of the primary antibodies overnight followed by a 2-h incubation with the secondary antibodies. Specimens were covered with Prolong Gold antifade mounting medium (Invitrogen, Carlsbad, CA, USA).

Analysis of identified somatic and visceral primary sensory neurons

Systemic random serial photomicrographs were taken with a Leica DMLB fluorescence microscope (Wetzlar, Germany) equipped with a Retiga 2000R digital camera (QImaging, Surrey, B.C., Canada). bWGA-positive neurons with visible nuclei were selected and analysis of retrogradely labelled neurons was performed by using ImageJ (NIH; Bethesda, Md., USA; IJ1.46r) image analysis software. Values are expressed as mean (standard deviation, SD). Statistical comparison of data was performed with the Fisher's exact probability test. A p value of ≤ 0.05 was considered significant.

Results

Retrograde labelling of spinal PSNs innervating somatic and visceral organs using bWGA

Injections of bWGA into the dorsal hind paw skin, the gastrocnemius muscle, the pancreas and the urinary bladder wall resulted in the labelling of numerous neurons in the segmental DRGs. We identified and analysed 147 neurons innervating the dorsal hind paw skin (three animals), 138 neurons innervating the gastrocnemius muscle (three animals), 174 neurons innervating the pancreas (four animals) and 225 neurons innervating the urinary bladder (four animals). The size frequency distribution of the labelled cells shows that bWGA identified small-medium sized neurons. The mean (SD) cross-sectional area of the labelled cutaneous, muscle, pancreatic and urinary bladder spinal PSNs amounted to 311.1 (43.4), 345.8 (55.9), 387.2 (49.4) and 339.2 (54.8) μm^2. We analysed the expression of the InsR and the TRPV1 in these retrogradely labelled neurons. Figure 1 illustrates target-identified retrogradely labelled neurons examined in this study showing InsR- and TRPV1-immunoreactivity.

InsR expression in retrogradely labelled spinal PSNs

Our quantitative data indicate that 22.4 (2.8) % of cutaneous, 21.8 (1.9) % of muscle, 54.1 (2.9) % of pancreatic and 53.4 (3.1) % of urinary bladder afferent neurons exhibited InsR immunoreactivity (Fig. 2). We found that there were no significant differences in the proportions of the InsR-immunoreactive neurons either between cutaneous and muscle afferents or between pancreatic and urinary bladder afferents. However, the statistical analysis revealed a highly significant difference between proportions of InsR-immunoreactive somatic and visceral afferent neurons ($p < 0.05$). We also assessed the proportions of TRPV1-immunoreactiveneurons in the bWGA-labelled InsR-immunoreactive neuron population. In the DRGs, 72.7 (3.4), 73.3 (2.6), 54.3 (2.9) and 57.1 (3.6) % of the bWGA-labelled InsR-immunoreactive neurons innervating the dorsal hind paw skin, the gastrocnemius muscle, the pancreas and the urinary bladder showed TRPV1 immunoreactivity, respectively (Table 1). We found no significant differences in TRPV1 expression among the four populations of neurons.

TRPV1 labelling of identified spinal PSNs

The large majority of identified PSNs expressed the TRPV1. We found that 63.1 (3.4) % of cutaneous, 62.5 (2.7) % of muscle, 66.9 (2.5) % of pancreatic and 65.0 (1.8) % of urinary bladder bWGA-labelled afferents displayed TRPV1 immunoreactivity (Fig. 2). There were no significant differences in the TRPV1 immunoreactivity among the subpopulations. We analysed InsR immunoreactivity of the retrogradely labelled TRPV1-positive neurons as well. In the dorsal root ganglia, 25.8 (2.2), 25.5 (2.4), 43.9 (2.3) and 46.6 (1.9) % of the bWGA-labelled TRPV1-immunoreactive neurons innervating the dorsal hind paw skin, the gastrocnemius muscle, the pancreas and the urinary bladder showed InsR-immunoreactivity, respectively (Table 2). We found that there were no significant differences in the proportions of the InsR-immunoreactive neurons either between the cutaneous and the muscle afferents or between the pancreatic and urinary bladder afferents. However, the differences between the cutaneous and pancreatic, the cutaneous and bladder, the muscle and pancreatic and muscle and urinary bladder PSNs were significant ($p < 0.05$).

Co-localization of the InsR with the TRPV1 in retrogradely labelled somatic and visceral spinal PSNs

Through analysing the co-expression of the InsR with the TRPV1 in retrogradely labelled cells, we found that 16.56 (0.6) % of cutaneous, 15.33 (1.1) % of muscle, 29.4 (1.8) % of pancreatic and 30.34 (2.1) % of urinary bladder afferent neurons exhibited both InsR and TRPV1 immunoreactivity,

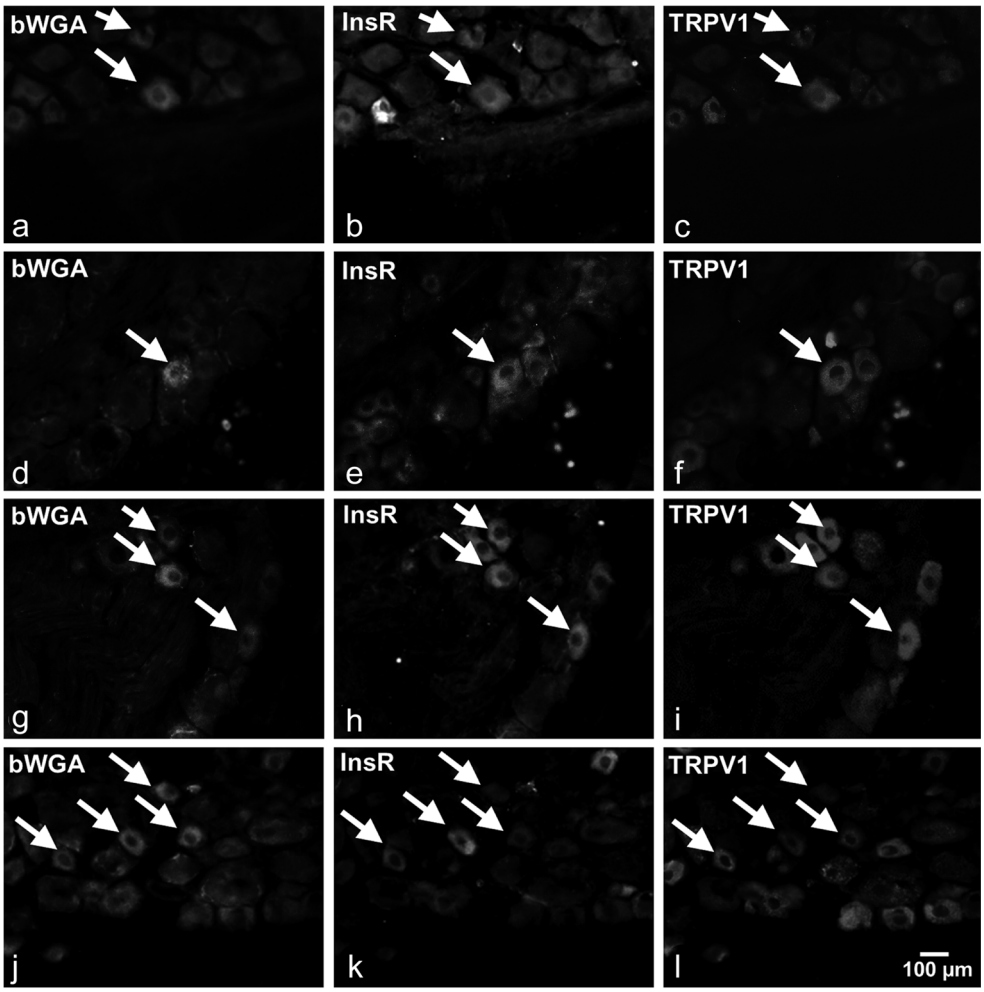

Fig. 1 Photomicrographs of biotin-conjugated wheat germ agglutinin (bWGA)-labelled primary sensory neurons innervating the dorsal hind paw skin (**a**, **b**, **c**), the gastrocnemius muscle (**d**, **e**, **f**), the pancreas (**g**, **h**, **i**) and the urinary bladder (**j**, **k**, **l**) showing immunoreactivities for the insulin receptor (InsR) and the transient receptor potential vanilloid type 1 receptor (TRPV1). The scale bar indicates 100 μm and applies to all photomicrographs

respectively (Fig. 2). There were no significant differences either between the cutaneous and muscle or the pancreatic and urinary bladder neurons. However, there were significant differences in the proportion of the InsR- and TRPV-immunoreactive neurons between the somatic and visceral PSNs ($p < 0.05$).

Discussion

Recently, it has been revealed that InsR is expressed in rat and mouse PSNS of unidentified target innervation territories (Sugimoto et al. 2002; Sántha and Nagy 2006; Baiou et al. 2007) and in rat pancreatic spinal and vagal PSNs (Lázár et al. 2018). Our present quantitative immunohistochemical study shows that up to 20% of the retrogradely labelled spinal PSNs innervating the dorsal hind paw skin or the gastrocnemius muscle express the InsR. The proportions of InsR-immunoreactive neurons were significantly higher, amounting up to 50%, among PSNs innervating visceral organs. Similarly to previous data, our present observations show that a relatively high proportion of PSNs expresses the InsR. In addition, we found differential expression of the InsR in PSNs innervating somatic and visceral organs, respectively. In PC12 cells, which share characteristics with nociceptive PSNs, the InsR has recently been shown to form a complex with TrkA, the receptor activated by NGF (Geetha et al. 2013). NGF is essential in the development of functional traits and neurochemical phenotypes of nociceptive PSNs (Snider and McMahon 1998). Moreover, the expression of the TRPV1 has been shown to be regulated by NGF (Michael and Priestley 1999). It is conceivable to suggest that through interacting with TrkA, the InsR contributes to the development, differentiation and functional characteristics of nociceptive PSNs.

In the present study, we also examined the TRPV1-immunoreactivity of the identified InsR-expressing PSNs. Our quantitative data revealed that up to 50% of identified InsR-expressing spinal PSNs exhibit TRPV1 immunoreactivity and in this respect, a significant difference between somatic and visceral neurons was not detected. Additionally, the analysis of the retrogradely labelled TRPV1-immunoreactive neurons shows that almost half of the visceral but only a quarter of the somatic TRPV1-immunoreactive PSNs exhibit the InsR.

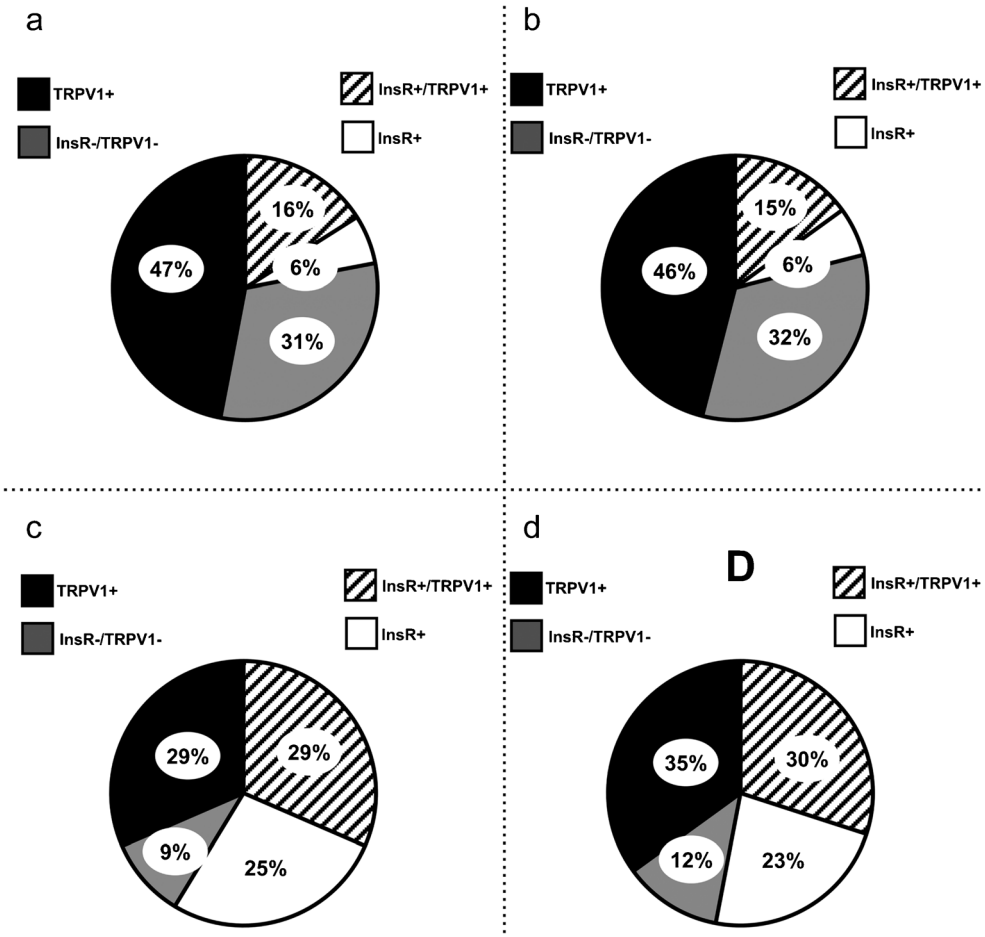

Fig. 2 Pie charts show the percentage distribution of populations of retrogradely labelled primary sensory neurons (PSNs) innervating the dorsal hind paw skin (**a**), the gastrocnemius muscle (**b**), the pancreas (**c**) and the urinary bladder (**d**). Pie charts were constructed by analysing the chemical phenotypes of 147 (**a**), 128 (**b**), 174 (**c**) and 225 (**d**) retrogradely labelled cutaneous, muscle, pancreatic and urinary bladder PSNs

Regarding the cell size and the TRPV1 expression pattern of the identified InsR-immunoreactive PSNs, it is highly likely that these neurons are nociceptive in function.

It has been demonstrated that systemic and local applications of insulin induce vascular changes mediated by the release of calcitonin gene-related peptide (CGRP) from capsaicin-sensitive PSNs (Salem and Dunbar 2002). It has also been revealed that InsR by modulating intracellular signalling systems may activate TRPV1-expressing PSNs (Heidenreich et al. 1990; Cesare et al. 1999; Chuang et al. 2001; Sathianathan et al. 2003). The present observations confirm the co-localization of the InsR with TRPV1 in PSNs (Sántha and Nagy 2006; Baiou et al. 2007; Lázár et al. 2018) and extend previous findings by showing differential co-localization of the InsR and the TRPV1 in somatic and visceral afferents. The relatively high co-expression rate of the InsR and the TRPV1 in spinal PSNs suggests a functional interaction between these receptors that may modify local tissue reactions effected by chemosensitive PSNs.

The expression of the InsR and the co-expression of the InsR with the TRPV1 may also bear important pathophysiological significance under conditions of unbalanced plasma insulin level. Insulin, through an action on TRPV1 signalling, may elicit a release of pro-inflammatory neuropeptides, such as CGRP (Salem and Dunbar 2002). This interplay may be important under hyperinsulinaemic conditions, such as the initial phase of type 2 diabetes mellitus. Furthermore, insulin-induced activation of TRPV1 may be

Table 1 Proportions of retrogradely labelled insulin receptor (InsR)-immunoreactive cutaneous, muscle, urinary bladder and pancreatic primary sensory neurons showing transient receptor potential vanilloid type 1 receptor (TRPV1) immunoreactivity

Target organ	Number of InsR-immunoreactive neurons	$TRPV1^+$ neurons (%)
Dorsal hind paw skin	33	72.7 (3.4)
Gastrocnemius muscle	30	73.3 (2.6)
Pancreas	94	54.3 (3.6)
Urinary bladder	119	57.1 (2.9)

Table 2 Proportions of retrogradely labelled transient receptor potential vanilloid type 1 receptor (TRPV1)-immunoreactive cutaneous, muscle, urinary bladder and pancreatic primary sensory neurons showing insulin receptor (InsR) immunoreactivity

Target organ	Number of TRPV1-immunoreactive neurons	$InsR^+$ neurons (%)
Dorsal hind paw skin	93	25.8 (2.2.)
Gastrocnemius muscle	86	25.5 (2.4)
Pancreas	116	43.9 (1.9)
Urinary bladder	146	46.6 (2.3)

essential in keeping physiological functions of capsaicin-sensitive sensory nerves (Sathianathan et al. 2003), which may be affected under hypoinsulinaemic conditions, such as in type 1 diabetes mellitus.

In recent years, many studies have focused on the role of TRPV1 in inflammatory processes of both the exocrine and the endocrine pancreas (Nathan et al. 2001; Razavi et al. 2006; Wick et al. 2006; Tsui et al. 2007; Iwasaki et al. 2013; Schwartz et al. 2013). More recently, it was also hypothesised that insulin, by modulating TRPV1 activation, has a pivotal role in the initiation of pancreatitis (Razavi et al. 2006; Tsui et al. 2007; Lázár et al. 2018). Our morphological findings demonstrating a high degree of co-localization of the InsR with the TRPV1 in neurons innervating the pancreas support this assumption. Similarly, diabetic cystopathy resulting in increased post-voiding residual volumes and enhanced urinary bladder capacity is accompanied by decreased bladder sensation and contraction secondary to damage of visceral afferent fibres in the urinary bladder wall (Kebapci et al. 2007). This assumption is supported by observations of increased bladder capacity and disturbances in urinary bladder reflexes and micturition after neonatal capsaicin treatment (Jancsó and Maggi 1987; Maggi et al. 1993), which produces a selective loss of nociceptive C-fibre PSNs (Jancsó et al. 1977; Jancsó and Maggi 1987). Our findings, showing that ~50% of TRPV1-immunoreactive neurons co-express the InsRs, suggest a possible modulatory influence of insulin on urinary bladder sensory nerves.

In conclusion, the present study demonstrates that a higher proportion of visceral PSNs express the InsR as compared to somatic ones and that TRPV1-expressing visceral afferent neurons show a higher InsR expression rate than somatic PSNs. Taking these morphological observations into consideration, it could be hypothesised that the TRPV1-dependent functions of visceral PSNs are more sensitive to changes in plasma insulin levels and these neurons may be more susceptible to an interplay of the InsR and the TRPV1 under pathological conditions. In addition, regarding the newly found co-localization of the InsR and the TRPV1in identified visceral afferent neurons and the functional interactions between these two receptors, a role of InsR-expressing visceral sensory nerves in the development of visceral inflammatory processes may emerge.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which studies were conducted (Ethics Committee for Animal Care at the University of Szeged—Directive 2010/63/EU of the European Parliament and of the Council on the Protection of Animals Used for Scientific Purposes and the Guidelines of the Committee for Research.)

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